South-East Asian and Australian Origins of Psittacine Beak and Feather Disease Virus of Captive African Psittacines in Saudi Arabia Indicate Interspecies Transmission

Ala'audin Hakami,[1] AusamaA Yousif,[2*] Mohamed Zaki,[3] Mahmoud Ismail,[4,5] Ahmed Al-Ali,[5] Abdu-Rahman Al-Ankari[5].

[1]Municipality of AhadAlmosarhah, Gizan, Saudi Arabia.
[2]Department of Virology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt.
[3]Department of Poultry Diseases, College of Veterinary Medicine, Cairo University.
[4]Department of Poultry Diseases, College of Veterinary Medicine, Kafrelsheikh University, Kafrelsheikh, Egypt.
[5]Department of Clinical Studies, College of Veterinary Medicine and Animal Resources, King Faisal University, Al-Ahsa, Saudi Arabia.

[2]

ABSTRACT

Clinical presentations of psittacine beak and feather disease virus (PBFDV) in Saudi Arabia are increasingly reported without laboratory diagnostic evidence of virus circulation. The objectives of this study were to investigate the presence of PBFDV in the Arabian Peninsula, and to identify its possible molecular origins. Total DNA was extracted from feather and blood samples from randomly selected 19 clinically-suspect and 153 apparently-normal birds collected from 10 locally-bred and imported Psittaciformes. Specimens were collected between 2008 and 2010. A replicase-associated protein (Rep) gene-specific PCR was used to amplify a 603 bp region of the viral genome. PBFDV was detected in 31.6% of clinical cases (3.5% of samples). Two positive samples were collected during 2008, three during 2009, and one during 2010. Positive samples were from clinical cases. Negative samples tested positive for bird rDNA. In positive cases, feathers not blood, consistently tested positive. Four of the positive samples were collected from African grey parrots (*Psittacuserithacus*), and two were from Indian ringnecks (*Psittaculaeupatria*). Nucleotide-based phylogenetic analysis clustered one African grey parrot PBFDV (Mira08) with South-East Asian and Australian isolates indicating interspecies transmission.

Key words: PBFDV, Blood, feathers, PCR, phylogenetic analysis.

INTRODUCTION

Psittacine beak and feather disease (PBFD) is a contagious, fatal viral disease that affects the beak, feathers, and immune system of wild and captive Old and New Worldpsittacines. PBFD is characterized by the roughly-symmetrical loss of feathers and feather abnormalities. Birds may also suffer from beak and claw deformities, eventually dying as a result of immunosuppression **(Kondiah*et al.*, 2006)**. The disease was first described in a wild population of sulphur-crested cockatoos (*Cacatuagalerita*) in Australia and is now recognized in many other bird species such as hooded parrots, lovebirds, king parrots, Mallee ring-necks and Port Lincoln parrots **(McOrist*et al.*, 1984).**

PBFD is caused by circovirus with a circular single stranded DNA genome, approximately 1.7–2.0 kb in size **(Bassami*et al.*, 2001)**. PBFD virus (BFDV) is an icosahedral enveloped virus 14–16 nm in diameter **(Ritchie *et al.*, 1989)**. PBFDV is transmitted horizontally and vertically **(Rahaus*et al.*, 2008)**. Definite viral diagnosis and research using conventional methodologies have been hindered due to the inability to propagate PBFDV in vitro so far **(Pass and Perry, 1985)**. The use of haemagglutination and haemagglutination inhibition is challenging. The polymerase chain reaction (PCR) is extremely useful for confirmation of BFDV DNA presence in whole blood or tissue samples with variable degrees of reliability **(Shearer *et al.*, 2009)**. PCR based diagnosis can be used for identification of both clinically suspect, and apparently normal carrier cases of PBFD .

There is a sustained increase in traffic of psittacine birds between SA and neighboring countries. Cases of feather loss and beak deformity in parrots suggestive of psittacine beak and feather disease (PBFD) are increasingly presented to avian clinics. Clinical records suggest that BFDV circulation has been ongoing for over 5 years despite the lack of laboratory-confirmed cases. In order to investigate this hypothesis, archival (from clinics) and fresh (from clinics, markets and farms) psittacine blood and feather samples from apparently healthy

and clinically affected birds were PCR-tested.

MATERIAL & METHODS

Samples, sample collection and preservation:

Archival and fresh samples from 19 clinically-suspect (Figure 1), and 153 apparently-normal birds were collected over a period of three years starting from 2008 and ending early 2010 to identify a possible virus introduction time into the Saudi environment. Bird species sampled belonged to 10 different species of imported or locally bred members of the order Psittaciformes in the Eastern and Southern provinces (Table 1). Samples included chest feathers as well as clotted whole blood. Blood was collected by either puncturing the wing vein using a sterile disposable needle, or by clipping one of the nails off the claw. Blood was collected directly into disposable 1.5 ml microfuge tubes (sample volumes ranged from 10-30 µl). Feather samples were plucked from the chest area or, collected from the birds' environment. Feathers were placed whole in sterile sample collection tubes and transported to the laboratory from remote locations in an ice box. In the laboratory both types of samples were properly labeled and stored in separate freezer boxes at - 20 ^{o}C until used for DNA extraction.

DNA extraction from blood and feathers:

Total DNA was extracted from blood samples using DNeasy Blood and Tissue kit (Qiagen, Cat.69504, USA) according to the manufacturer's instructions. DNA from feather samples was extracted using JETQUICK Tissue DNA Spin Kit according to the manufacturer's instructions(Cat. No. 450050, GENOMED GmbH, Germany), with some modifications for feather tip processing. Briefly, feathers were allowed to thaw at RT before processing. Distal, differentiated portions were cut off and discarded. The growing tips were retained for DNA extraction. Tips were cut into small pieces using a sharp scalpel on a fresh piece of disposable aluminum foil. Cut tips were then crushed using the broad end of the scalpel blade. Crushed tips were transferred into fresh microfuge tube, lyses buffer was added, and the procedure was completed according to the manufacturer's instructions. DNA extracts were preserved at -80 ^{o}C until testing.

The quantity and quality of extracted DNA was determined prior to PCR setup. Concentration and purity was determined spectrophotometrically using SmartSpecTM Plus spectrophotometer (BioRad, Hercules, CA, USA).

PCR:

A 603 nt fragment (Nucleotides 178-780) of the PBFDV Rep protein gene was amplified using the forward and reverse primers previously described: Forward 5'-TTAACAACCCTACAGACGGC GA-3' and Reverse 5'-GGCGGAGCATCTCGCAATAA G-3' **(Ritchie*et al.*, 1998)**. The presence of avian DNA, and the absence of inhibitors in the extracts was assessed by amplification of a 530-nt fragment of the avian mitochondrial ribosomal RNA gene using the previously described primers L-12SA 5'-AAACTGGGATTAGATACCCC ACTAT-3' and H-12SB2 5'-CTTCCGGTACACTTACCTTGT TACGAC-3' **(Kocher*et al.*, 1998)**. Amplification reactions were carried out using the HotStarTaq® Plus Master Mix PCR kit (QIAGEN, Hilden, Germany). Each reaction tube contained 1 unit of HotStarTaq® Plus DNA polymerase in 1× PCR buffer containing (KCl and $(NH4)_2SO_4$), 200 uM of each dNTP, 0.5 uM of each of the forward and reverse primers, 1× Coral Load dye mix, and 3 µl of the sample DNA and controls. The reaction tubes were centrifuged at 2000×g briefly, and placed in a BioRadMyCyclerTM thermal cycler (BioRad, Hercules, CA, USA).

Thermal cycling conditions for amplification of viral and avian DNA targets genes were: an initial denaturation step (95 ^{o}C for 4 min), 38 amplification cycles (95 ^{o}C for 30 s, 54.5 ^{o}C for 30 s, and 72 ^{o}C for 50 sec), and a final extension step (72 ^{o}C for 5 min). After amplification, 15 µl of each sample were analyzed by electrophoresis on a 1.2% agarose gel stained with 0.5 µg/ml ethidium bromide. DNA bands were visualized by UV irradiation in a Gel Doc XR gel documentation system (BIO-RAD laboratories, Milan, Italy).

Sequencing and sequence analysis:

PCR products were purified from gels using QIAEX® Gel Extraction Kit (QIAGEN Sciences, MD, USA). Purified PCR products were sequenced in both orientations by the dideoxy chain-termination

method using the amplification primers described above. Twenty reference PBFDV sequences were used for the alignment and phylogenetic analysis (Table 2). Nucleotide multiple sequence alignments and evolutionary analyses were conducted in MEGA5.15 The evolutionary history was inferred using the Neighbor-Joining method **(Saitou andNei, 1987)**. Phylogeny was tested using the bootstrap test (1000 replicates)**(Felsenstein, 1985)**. The evolutionary distances were computed using the Maximum Composite Likelihood method **(Tamura*et al.*, 2004)**.

RESULTS

Pure DNA extracts were obtained from blood and feathers

Blood and feather samples yielded DNA concentrations from 1-11 µg/ml (0.2-2.2 µg/sample). DNA extracts were pure (OD260/OD280 values ranging from 1.7-2.5). .

Table (1): Psittaciformessampled, and sample information.

Host Species	Common Name	Samples	Sampling Location				Collection Date			Type		Clinical Status	
			Clinic	Market	Farm	Store	2008	2009	2010	B	F	S	N
Psittacuserithacus	**African Grey**	49	12	12	9	16	18	18	13	14	35	16	33
Psittaculaeupatria	**Indian ringneck**	28	-	9	5	14	1	19	8	13	15	1	27
Psittaculaeupatria	**Local ringneck**	13	-	12	1	-	1	12	-	6	7	1	12
Agapornisroseicollis	**Lovebird**	42	10	18	14	-	-	24	18	12	30	-	42
PsittaculaKrameri	**Parakeet**	4	-	-	-	4	-	-	4	1	3	-	4
Nymphicushollandicus	**Cockatiel**	13	1	1	1	10	1	8	4	-	13	1	12
Ara macaw	**Macaw**	4	-	1	1	2	-	2	2	1	3	-	4
Cacatuagalerita	**Cockatoos**	3	-	3	-	-	-	3	-	1	2	-	3
Agapornisfisheri	**Fisher's**	10	-	2	-	8	1	5	4	2	8	-	10
Amazona autumnalis	**Amazon**	3	3	-	-	-	-	-	3	-	3	-	3
Poicephalus senegalus	**Senegal parrot**	3	-	2	-	1	1	2	-	1	2	-	3
Totals		**172**	**26**	**60**	**31**	**55**	**23**	**93**	**56**	**51**	**121**	**19**	**153**

B: Blood. F: Feather. S: Suspected case: Birds showing deformities in the beak and/or feathers. N: Apparently normal

Table (2): Reference PBFDV sequences form used in sequence analysis.

Host species	Common name	Host Location	GenbankAcc. #
Melopsittacusundulatus	Budgerigars	Japan	AB277748.1
Melopsittacusundulatus	Budgerigars	Japan	AB277751.1
elopsittacusundulatus	Budgerigars	Japan	AB277750.1
Melopsittacusundulatus	Budgerigars	Japan	AB277746.1
Poicephalusrobustus	Cape parrot	South Africa	AY450438.1
Agapornisnigrigenis	Black-cheeked lovebird	South Africa	AY450442.1
Agapomisroseicollis	Rosy-faced lovebird	Australia	AF311296.1
Psittacuserithacus	grey parrot	Portugal	EU810207.1
Psittacuserithacus	grey parrot	Portugal	GQ329705.1
Psittacuserithacus	grey parrot	South Africa	AY450443.1
Psittacuserithacus	grey parrot	Portugal	EU810208.1
Araararauna	Macaw	Thailand	FJ685980.1
Proboscigeraterrimus	Cockatoo	Thailand	GU015022.1
Psittaciformes	?	USA	AF071878.1
Psittacuserithacus	grey parrot	South Africa	AY450435.1
Cyanoramphusnovaezel andiae	red-fronted parakeets	New Zealand	GQ396652.1
Nymphicushollandicus	Cockatiel	Japan	AB514568.1
Nymphicushollandicus	Cockatiels	Australia	EF457975.1
Cacatuatenuirostris	Corella	Australia	AF311297.1
Cacatuagalerita	Sulfur-crested cockatoo	Australia	AF311301.1

PBFDV sequences were amplified by PCR

Testing was started on archival and fresh suspect samples showing the typical clinical presentation of PBFD with the hope of finding positive samples because it was decided to avoid introduction of standard positive materials into the laboratory to avoid the possibility of contamination of the tests and/or the environment with a novel virus. The archival blood sample collected in 2008 produced the expected size PCR product in gel analysis (603 bp) (Figure 1A). Since, samples identified were at the lower limit of the test detection capacity. Serial dilution of positive samples did not result in PCR amplification. This lead to the use of a PCR positive control for the PBFDV PCR assay using diluted PCR products from the archival sample. PCR products diluted 1:1000 were used as positive controls after sequence confirmation to avoid introduction of infected material into SA and the laboratory in which the test was being established (Figure 1A). Avian mitochondrial ribosomal RNA gene amplification was done prior to further PBFDV PCR testing to insure that DNA concentration and quality were not factors in PBFDV PCR results (i.e. to exclude false negative results). Successful amplification of 530 bp PCR products was achieved from tested avian DNA extracts at the lower and higher DNA concentration values (Figure 1B).

A total of 6 samples were PCR-positive (3.48%) (Figure 1C); including the archival sample used as positive control. Two positive samples were collected during 2008, 3 during 2009, and only 1 during 2010. All positive samples were collected from clinically suspect birds. Four of the positive samples were collected from Grey parrots (*Psittacuserithacus*). Two of the samples were from ringneck birds (*Psittaculaeupatria*) (one Indian and one local). It was interesting to notice that in the two birds from which both blood and feathers were collected, only feather samples were positive to PBFDV. Only two blood samples were positive to PBFDV. All positive samples were collected from the Eastern province (Table 3).

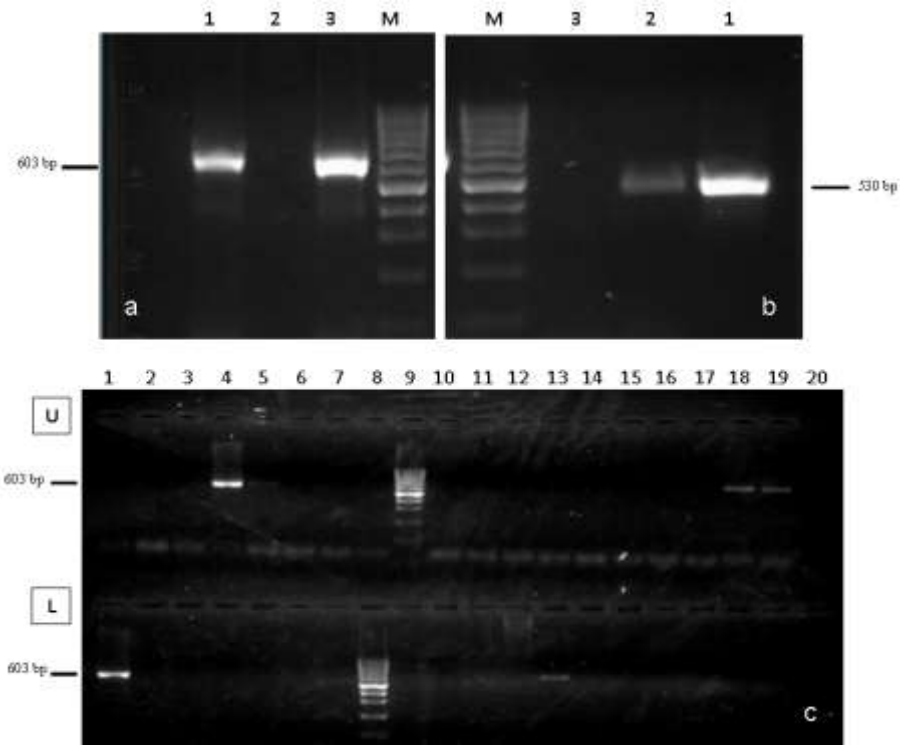

Figure (1): Detection of PBFDV DNA in avian blood and feather DNA extracts. [A] PBFDV Positive controls. DNA from 10 µl of avian blood collected from a bird showing clinical PBFD were extracted using DNeasy Blood & Tissue Kit (QIAGEN Sciences, Maryland, USA) and PCR-tested using the replicase-associated PBFDV gene PCR primers (Lane 1A) **(Ritchie *et al.*, 2003)**. A 10-3 dilution of the positive PBFDV PCR product was PCR amplified using the above primers to be used as the positive control in further testing (Lane 3A). The no template control is in lane 2A. Lane M is the VC 100 bp plus molecular weight marker (Vivantis Technologies Sdn. Bhd., Selangor DarulEhsan, Malaysia). [B] Evidence of the absence of false negative results. A 530-nt fragment of avian mitochondrial 12S ribosomal RNA was amplified from DNA extracts using previously described primers [4]. Amplification was achieved at the higher (2.2 µg) (Lane 1B), and lower (0.2 µg) (Lane 2B) DNA yield levels. The no template control is in lane 3B. Lane M is the VC 100 bp plus molecular weight marker (Vivantis Technologies Sdn. Bhd., Selangor DarulEhsan, Malaysia). [C] PCR screening of select avian DNA extracts for the presence of PBFDV DNA. Lanes 4 upper (4UC), and 1 lower (1LC) are positive controls. The upper lanes 5, 18, and 19, and lane 13 of the lower section of figure 1C are positive. Lanes 9 in the upper section and lane 8 of the lower section of figure C are the VC 100 bp plus molecular weight marker (Vivantis Technologies Sdn. Bhd., Selangor DarulEhsan, Malaysia).

Sequence analysis of amplified PBFDV indicate Australian and South East Asian origins

The PBFDV DNA sequence used as a positive control was designated MIRA08. Partial ORF V1 sequences representing a portion of the Rep gene were submitted [GenBank: HM565916]. Multiple alignments of Mira08 and 20 reference PBFDV Rep gene nucleotide sequences showed that multiple nucleotide substitutions were observed along the length of the studied Rep gene. Nucleotide substitutions C9A, C13G, C33A, C88A, A100G, C101A, A/G119T, and A195G, were unique to Mira08. Sequence identity percentages between Mira08 and reference strains used in this study ranged from 83.1% to 92.8%. The lower identity percent was obtained with a newly proposed genotype in Japan [GenBank: AB277751.1]. The higher identities were observed with two Australian strains that clustered with Mira08 in phylogenetic analysis (one from a sulfur-crested cockatoo [GenBank: AF311301.1] and the other from corella [GenBank: AF311297.1]). Two Japanese isolates from budgerigars [GenBank: AB277746.1 and GenBank: AB277750.1] also clustered with Mira08 (Figure 2).

Table (3): PBFDV PCR-positive sample detailes.

Host species	Host common name	Sampling Location	Date	Clinical status	Blood	Feather
Psittacuserithacus	Grey parrot	Avian clinic	2008	S*	+	NT
Psittacuserithacus	Grey parrot	Avian clinic	2008	S	+	NT
Psittacuserithacus	Grey parrot	Al-qatif bird market	2009	S	-	+
Psittacuserithacus	Grey parrot	Al-ahsa bird market	2009	S	NT	+
Psittaculaeupatria	Local ringneck	Al-qatif private farm	2009	S	NT	+
Psittaculaeupatria	Indian ringneck	Al-ahsa bird market	2010	S	-	+

*S: Clinically suspected case. NT: not tested

DISCUSSION

PBFD is the most common viral disease of wild and captive Psittaciformes. PBFDV has the potential to become a major threat

to all species of wild parrots and modern psittacine aviculture due to the increasing international legal and illegal bird trade. Cases of PBFD have now been reported on all continents in at least 40 member species of Psittaciformes**(Kondiah*et al.*, 2006)**, and this is likely to increase. PBFDV was not previously reported in SA, and this is the first laboratory-confirmed report of the virus in the Arabian Peninsula.

The number of samples tested was deemed adequate to detect infected birds in target populations because of the reported high prevalence of the virus in psittacine populations in avicultures in other parts of the world **(Shearer *et al.*, 2008)**; a survey in South Africa investigated only 161 bird samples and yielded 75 positive samples (46.5%) **(Kondiah*et al.*, 2006)**.

Testing was started on archival and fresh suspect samples showing the typical clinical presentation of PBFD with the hope of finding positive samples because it was decided to avoid introduction of standard positive materials into the laboratory to avoid the possibility of contamination of the tests and/or the environment with a novel virus. This situation produced a risk of missing positive samples due to low DNA extraction levels or the presence of inhibitors. However all samples were positive for Avian mitochondrial ribosomal RNA gene indicating the absence of inhibitors and the presence of DNA in extracts in amounts suitable for PCR amplification.

The archival blood sample produced the expected size PCR product in gel analysis (Figure 1A). Since, samples identified were at the lower limit of the test detection capacity. Serial dilution of positive samples did not result in PCR amplification. This lead to the use of a PCR positive control for the PBFDV PCR assay using diluted PCR products from the archival sample. PCR products diluted 1:1000 were used as positive controls after sequence confirmation to avoid introduction of infected material into SA and the laboratory in which the test was being established (Figure 1A).

In this study, bird owners were reluctant to provide blood samples because they feared that would sicken their birds. Feather samples were a suitable alternative. In the two birds from which both blood and feathers were collected, only feather samples were positive to

PBFDV. Only two blood samples were positive to PBFDV.

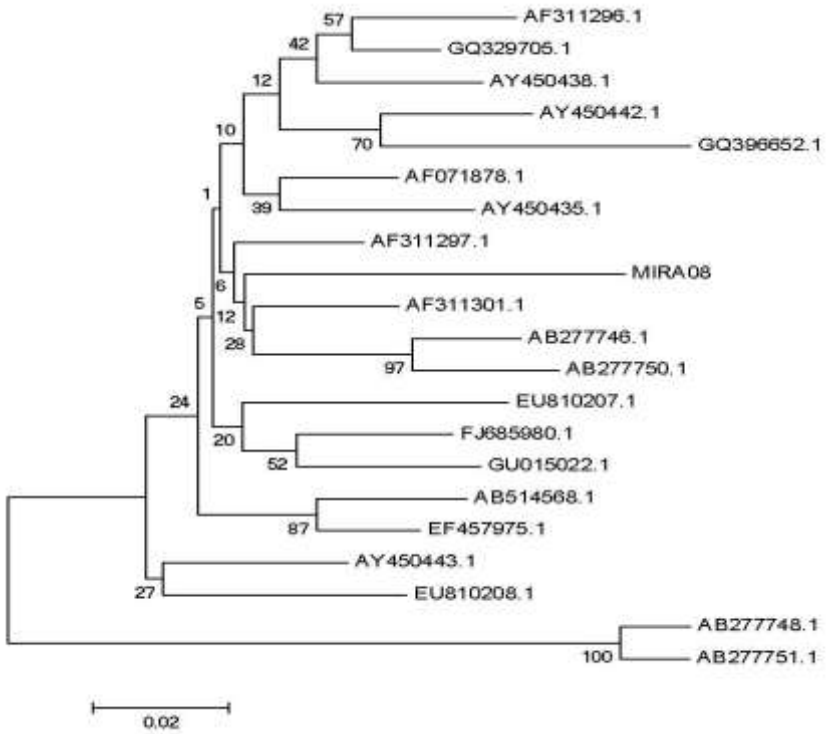

Figure (2): Phylogram constructed using partial replicase-associated PBFDV gene of the PBFDV Mira08 [GenBank: HM565916] and 20 reference PBFDV sequences from GenBank (Table 2). The evolutionary history was inferred using the Neighbor-Joining method, and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA5. Mira08 was most closely related to the sequences of two PBFDV isolates from Australia and two isolates from Japan (Table 2).

Surprisingly, PBFDV was not detected in any of the apparently normal birds. Latently infected birds can cause severe environmental contamination. Latent infections have been reported to be the majority of infections in captive psittacine populations **(Bert et al., 2005)**. The small number of positive samples detected in this relatively large sample size and the absence of detectable latent infections may be attributed to two causes: 1) Intensive breeding of members of Psittaciformes in SA is a relatively new practice; which would reduce the chance of horizontal and vertical transmission of the virus reported by others **(Rahaus*et al.*, 2008)**. 2) Birds belonging to Psittaciformes imported into SA are from the new world; which suffers a lower prevalence of the virus **(Bert *et al.*, 2005)**. 3) Virion numbers per feather tip in apparently normal carriers may be lower than that in clinical PBFD cases, despite the apparent importance of feather sample testing. Taken together, the prevalence reported in this study should be taken with caution as an indication of the prevalence of the disease in SA, and a more detailed investigation into the prevalence of the disease in SA is needed.

Another interesting finding is that positive samples were only found in 2 out of 10 bird species investigated. The ring-neck is bred easily in SA. The African grey parrot is expensive and owners are willing to spend money to try to treat infected birds and/or try to sell it to unsuspecting buyers. Therefore, it is possible that the virus was detected in both but not in other species where breeding is less likely, and/or the cost is low enough to discourage taking care of sick birds; in such cases, sick birds would have been discarded by owners.

Clustering of two Australian and two Japanese strains with Mira08 in phylogenetic analysis suggests that Mira08 probably originated from South East Asia or Australia. Given the rapid rate of evolution of avian circoviruses**(Varsani*et al.*, 2010)**, the unique mutations observed in PBFDV Mira08 may indicate that the virus was circulating in the environment for a few years prior to 2008. This hypothesis may be supported by the fact that PBFDV Mira08 was from a sample collected from an African grey parrot traded locally, and not

Australian or South-East Asian birds. There is a possibility that other positive birds could have acquired the infection due to exposure within the SA bird markets or avian farms. This study recommends further PCR testing of traded Psittaciformes species to suppress fraud and to exclude PBFDV during differential diagnosis in clinics.

ACKNOWLEDGEMENTS

The work was partially funded by the Deanship of Scientific Research, KingFaisalUniversity. Sequencing was provided free of charge by to Dr Ahmed Al-Qahtani, Head of Molecular Virology and Infectious Diseases Section, King Faisal Specialist Hospital and Research Center, Riyadh, SA.

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